Bacterial Endosymbionts: Master Modulators of Fungal Phenotypes

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INTRODUCTION
From the saprotrophs that decay plant material to the pathogens and mutualists that shape plant population dynamics at local and regional scales, fungi are major drivers of ecosystem health, plant productivity, and sustainability in all major biomes (1–5). The ecological roles of such fungi are driven by their own genomic and epigenetic architecture, as well as that of their hosts, often with strong influences from the environmental context in which such interactions occur (1–9).

The environmental context of fungal biology has long been defined in terms of abiotic factors, including those that impact fungal growth and gene expression directly and those that influence the health, growth, structure, or defenses of hosts or substrates with which fungi interact (Fig. 1). Increasingly, however, it is clear that these environmental contexts also include biotic components—that is, additional, interacting species that directly or indirectly alter the outcomes of fungus-host associations. Foremost among these in terms of major effects are endohyphal microbial symbionts, including viruses and bacteria that occur within living fungal hyphae and can profoundly alter fungal phenotypes (i.e., endofungal microbes or microbial endosymbionts) (10–13).

Phenotypic modulation of fungi by endohyphal microbes can cause major shifts in fungal substrate use, enzyme production, thermotolerance, virulence, and the early phases of symbiotic establishment. Much like horizontal gene transfer (HGT) can alter diverse aspects of an organism’s phenotype in ecological time, so too can acquisition (or loss) of a microbial symbiont change a virulent fungal pathogen to an avirulent lifestyle, alter the reproductive strategy of the fungus from sexual to asexual, increase fungal secretion of hormones and other signaling molecules relevant to plants, or broaden the niche space and competitive ability of fungi in ways that change the balance of fungal community dynamics (see references 11–19). In these ways endohyphal microbes are important not only in ecological time, but also in the evolution of fungi and reciprocal coevolutionary responses from their hosts (Fig. 1). Thus, understanding the mechanisms underlying symbiotic modulation of fungal phenotypes by microbes has the potential to inform not only fungal ecology but also the trajectory of fungal evolution, especially with regard to convergent phenotypes or ecological modes (e.g., endophytism) for which genomic markers and evolutionary shifts remain elusive. Such questions, paired with growing interest in applications of microbial endosymbionts of fungi, frame an exciting time as mycologists define myriad factors that shape fungal functional traits in short-term ecological snapshots and over evolutionary time.

Broadly defined under de Bary’s conceptualization as “the living together of unlike organisms” (20), symbioses between fungi and endohyphal microbes encompass mutualistic, parasitic, and commensal interactions, many of which may be context-dependent in terms of their importance or effects. The best known microbes that occur as endohyphal symbionts represent two domains of life. The first are acellular microbes such as mycoviruses. Mycoviruses (viruses that infect fungi) usually are double-stranded RNA viruses (ca. 70% of known mycoviruses), positive-sense, single-stranded RNA viruses (ca. 30%), and rarely, geminiviruses (21).
Viruses clearly are important in fungal biology (22–25). Their effects include hypovirulence of pathogens such as Cryphonectria parasitica, Sclerotinia sclerotiorum, Botrytis cinerea, and Fusarium graminearum (26–30); disease in various commercially important Agaricomycetes (31, 32); thermotolerance of plants via infection of the fungal endophyte Curvularia protuberata (33); and interference with the reproduction of other mycoviruses (34). Studies over the past 50 years have revealed that mycoviruses are ubiquitous in fungi (35, 36), with modes of transmission primarily via hyphal anastomoses and both sexual and asexual spores (but see reference 37 for an example of transmission via a mycophagous insect). Mycoviruses and their importance in fungi represent an exciting frontier for future research relevant to all aspects of fungal biology, and the themes structuring such research (i.e., effects on host phenotypes and gene expression, interaction with cellular defenses, modes of transmission, and roles in fungal ecology and evolution) are being investigated in parallel with studies of the other well-documented and diverse endosymbionts of fungi, in the domain Bacteria.

Much like other eukaryotes, fungi interact closely with bacteria. These interactions occur in the mycelosphere (the area around the mycelium with which the fungus interacts), on living hyphae (i.e., on the myceloplane), and within living structures such as hyphae (and in some cases spores; reviewed in reference 16). Those bacteria that occur within living hyphae are endofungal bacteria or endohyphal bacteria (EHB), broadly parallel to the concept of fungi occurring as endophytes of plants. These bacteria are grouped functionally based on their observation within hyphae, but their names do not imply obligate endosymbiotic lifestyles or prescribed benefits or costs to hosts. Instead, these phylogenetically diverse bacteria appear to have converged upon the capacity to infect fungal hyphae (38), together encompassing a spectrum of facultative to obligate associations, diverse localizations within fungal cells, and a range of outcomes of their associations.

Our thesis is that understanding the contributions of these EHB to fungal biology is a major frontier in studies of the fungal kingdom (16, 38–42). Their study is particularly exciting at present given the potential to explore new fungal strains and well-established model systems with high-throughput phenotyping and front-line molecular and computational methods in metabarcoding, genomics, and metatranscriptomics (19, 41–44). Our goals in this chapter are to provide a historical perspective on EHB as a means to frame a functional classification system for these bacterial associates of fungi, to summarize their functional importance and related genomic traits, and to highlight emerging methods by which EHB can be studied, pointing to emergent questions regarding the role of bacterial endosymbionts in fungal biology.

**HISTORICAL PERSPECTIVES**

Often present in symptomless mycelia, EHB inhabit diverse fungi representing at least the Mucoromycota (*sensu* reference 45: Mortierellomycotina, Mucoromycotina, Glomeromycotina) and Dikarya (Basidiomycota and Ascomycota) (38, 46–49). Most fungi documented as hosts of EHB are plant-associated, including arbuscular and ectomycorrhizal fungi, plant pathogens, saprotrophic fungi, soilborne fungi that interact with seeds,
and endophytes of tissues such as roots and leaves. Some clinically important fungi also harbor EHB, though they do not appear to play a major role in pathogenesis in human and animal systems (50–52).

Although it is not surprising that like many eukaryotes, fungi host bacterial endosymbionts, the discovery of their prevalence and their potential to shape major plant-fungi interactions has captured recent interest. The seminal discovery in 2005 that pathogenicity of a major plant pathogenic fungus, *Rhizopus microsporus*, was driven by an EHB (14) brought classical research on “bacteria-like organelles” (BLOs) (53) to the fore of plant pathology and mycology alike. Now, bacterial genomics, microscopy, and molecular biology are providing insights into the endohyphal lifestyle and the mechanisms by which such bacteria shape fungal traits.

EHB were first described in arbuscular mycorrhizal fungi in the 1970s (54, 55), although their status as bacteria was not confirmed until they were imaged by electron microscopy (53). Their discovery was complemented by the description of the cyanobacterium *Nostoc punctiforme* living within the fungus *Geosiphon pyriforme* (Glomeromycota) (see 15, 56–59). Most classical mycology texts rarely mention bacteria except to differentiate them from fungi (e.g., 60–63, as also observed in reference 64). However, Wolf and Wolf frequently reference bacteria-fungi interactions, referencing “associative interactions” and characterizing fungi as gregarious in terms of their associations with prokaryotes (65). Notably, Wolf and Wolf also note that “pure cultures” of fungi in nature are “either non-existent or else occur as miraculous oddities” (65), speaking presciently to our current understanding of the complex associations that frame many aspects of microbial and fungal ecology.

In the 1970s several studies established that BLOs were present in diverse fungi. Mosse noted the presence of self-replicating bacteria in spores of *Endogone*, speculating that BLOs observed there might be spores of Actinobacteria (66). Protzenko also observed a bacterium or BLO in an endotrophic mycorrhizal fungus of pea, noting that the microbe’s cell wall was located in close proximity to that of its host and stating that no morphological response of the fungus was observed (54). Microbial associates were observed in Ascomycota as early as 1973, when intracytoplasmic organisms (“probably bacteria”) were recorded in *Scutellinia*, a genus of saprotrophs (67).

In most of these cases early observations were by microscopists, with MacDonald et al. noting that a major challenge in the further study of BLOs was that they appeared to be unculturable (68). Perhaps for this reason studies contemporaneous with the early description of BLOs focused on other bacteria-fungi associations despite compelling evidence of unusual microbial- or microbe-like structures in fungal hyphae. For example, the last decades of the 20th century were particularly rich in studies regarding mycorrhizal helper bacteria (reviewed in reference 69; see also reference 16), bacterial ectosymbionts of fungi (70, 71), interactions between cyanobacteria and lichen-forming fungi (72), bacterial pathogens of fungi (73, 74), the importance of mixed-microbial fermentations in foods (64, referencing 75), and antagonism between fungi and bacteria. Scannerini and Bonfante-Fasolo summarized the state of the field with respect to BLOs and endosymbiotic bacteria in fungi in general: “the available information remains scanty” (76).

Even so, Scannerini and Bonfante-Fasolo pointed to ca. 10 published and unpublished studies highlighting the structural classification of BLOs and endosymbiotic bacteria in arbuscular mycorrhizal fungi, providing evidence that these “endocytobionts” could be observed in spores, hyphae, arbuscules, and other structures (76). Moreover, they diagnosed the existence of two types of endocytobionts in fungal tissue: (i) rod-shaped bacteria with visible cell walls, nucleoids, and ribosomes, typically enclosed in vacuoles, observed in *Glomus* and an unidentified arbuscular mycorrhizal fungus (68, 77, 78), and (ii) BLOs, which lack cell walls, have a coccoid form, and are localized in the cytoplasm of diverse arbuscular mycorrhizal fungi. MacDonald and Chandler reported that such BLOs typically could be found near the nucleus or mitochondria of fungal cells, although their placement varied (53). Scannerini and Bonfante-Fasolo concluded that these BLOs resemble the vertically transmitted bacterial endosymbionts of some insects (76).

Enabled by these pioneering studies, a groundswell of interest in symbioses, and the combination of new analytical methods in visualization and molecular biology, a renewed focus on bacterial endosymbionts of fungi has emerged over the nearly 3 decades since Scannerini and Bonfante-Fasolo’s review (76). Reconstruction of this phase in the study of bacterial endosymbionts is somewhat challenging because terminology has changed over time (i.e., endocytobionts, endocellular bacteria, endofungal bacteria, endobacteria, bacterial endosymbionts and endosymbiotic bacteria, and EHB). However, literature searches (e.g., in the Web of Science, early 2017, with these terms in Boolean arrays) point to a growth in publication rate from 1996 onward, especially over the past 10 years.
In 1996, one paper highlighted the presence of ca. 250,000 BLOs in a single spore of the arbuscular mycorrhizal fungus *Gigaspora margarita* and used 16S rRNA sequencing to identify bacteria within living fungi as members of the Gram-negative bacterial genus *Burkholderia* (Betaproteobacteria; now recognized as *Paraburkholderia* and hereafter referred to as such [46, 79]). By 2000, such studies included surveys of diverse arbuscular mycorrhizal fungi and revealed phylogenetic relationships among *Paraburkholderia* associated with various Gigasporaceae (80). Tools such as *in situ* hybridization further enabled visualization of symbiotic bacteria in spores and hyphae (e.g., of *G. margarita* [80]). Subsequent studies moved rapidly to address the functional significance of such bacteria, drawing from genomics to show that a *Paraburkholderia* in *G. margarita* has nitrogen fixation genes (81) and identifying regions coding for particular proteins (e.g., a methyl accepting chemotaxis protein [82]).

The first major papers on other fungal systems emerged with the 2005 study of *R. microsporus* (Mucoromycotina), wherein a strain of *Paraburkholderia* was shown to produce rhizoxin, a macrocyclic polyketide metabolite and phytotoxin critical to pathogenicity associated with rice seedling blight (14). By 2010, endohyphal *Burkholderiaceae* also were reported from Mortierellomycotina (83), highlighting the prevalence of this bacterial family across the Mucoromycota. Contemporaneously, *Alphaproteobacteria* were identified in plant-associated mycelium of the ectomycorrhizal basidiomycete *Laccaria bicolor* (84), demonstrating that diverse lineages of *Proteobacteria* can occur as endo-fungal symbionts and indicating that septate hyphae such as those in the Basidiomycota did not preclude establishment of EHB. A second *Alphaproteobacteria*, *Rhizobium radiobacter*, was identified subsequently as relevant to the establishment of endophytic symbioses by the basidiomycete *Piriformospora indica* (Sebacinales), which affiliates with roots of barley and other hosts (85).

Since that time, studies of EHB have widened in two main ways. First, the combined approaches of molecular biology, genomics, and computational biology have led to the identification of the coccoid BLOs in Glomeromycotina that had been observed for decades by microscopy alone. Now known as *Mollicutes*-related endobacteria and placed phylogenetically in the *Tenericutes*, these EHB are recognized as widespread symbionts of arbuscular mycorrhizal fungi (86, 87). They exhibit within-host genetic variation, small genomes, and complex genomic and evolutionary histories (41, 42, 88).

Second, the study of EHB now has extended to the Ascomycota, the most species-rich phylum of fungi. In the first studies evaluating phylogenetically diverse Ascomycota for EHB, fungi occurring as foliar endophytes were shown to harbor not only many lineages of *Proteobacteria* but also diverse Gram-negative and Gram-positive bacteria from the *Bacteroidetes* and *Firmicutes*, respectively (38, 49). As in other groups of fungi, visualization of these EHB was facilitated by LIVE/DEAD staining (i.e., LIVE/DEAD BacLight Bacterial Viability Kit, Invitrogen [38]) and by more precise methods such as fluorescent *in situ* hybridization (Fig. 2) and bacterial transformation for fluorescence (Fig. 3). Recent work has addressed the phylogenetic diversity of EHB (Fig. 4) (see 49), their significance with regard to fungal thermotolerance, production of plant hormones and activity of plant cell wall-degrading enzymes (Fig. 5) (11, 17), their alteration of fungal growth and plant tissue degradation (Fig. 6), their capacity to influence early stages of symbiotic establishment between endophytes and hosts (Fig. 7), their variation among closely related fungi that infect seeds as opposed to leaves (49), their ability to alter carbon substrate use by a
seed-associated fungus in vitro (19), and their genomic features (Fig. 8) (43).

Together, ca. 100 studies since 1996 have explored the biological, genomic, biochemical, evolutionary, ecological, and functional traits of EHB, painting a picture quite different than that encapsulated by the axenic approach to fungal biology (per reference 65): one of widespread, diverse, and important symbioses that frame fungal traits in cryptic ways. Emergent themes are that EHB are common in fungi, that they likely represent convergence in the general ability to live within fungi but do so in diverse ways with differing outcomes,

Figure 3  (A) Fluorescence microscopy (400×) reveals successful reintroduction of the class 3 EHB Luteibacter sp. 9143 with the tdTomato construct into hyphae of Pestalotiopsis sp. 9143. (B) The same image under phase contrast. (Images reprinted with modification from reference 48). (C) Healthy and apparently axenic colony of Pestalotiopsis sp. 9143 in which EHB are present but not visible without microscopy. (D) Conidium of Pestalotiopsis sp. 9143. Although EHB are widespread in the culture that produced such conidia, no conidial transmission (i.e., no vertical transmission) has been detected.
Figure 4  Phylogenetic relationships of selected class 2 EHB (black squares) associated with Mucoromycota, class 3 EHB (bold text) in the Proteobacteria and Firmicutes that associate with fungal endophytes in the Ascomycota, known bacteria (regular font), and bacterial endophytes (black circles). For fungal endophytes hosting class 3 EHB, taxon labels indicate the fungal genus, the plant species from which these fungi were obtained (Cupressus arizonica, Juniperus deppeana, Juniperus scopulorum, Platycladus orientalis), the location in which the host tree was growing (NC, North Carolina; UA, University of Arizona Campus Arboretum, Tucson, Arizona; CHU, M, and MTL, montane regions of Arizona), relevant GenBank accessions, and bacterial genotype groups (operational taxonomic units) based on 97% similarity of bacterial 16S rRNA (38). Colored labels indicate genome size and GC content for class 2 EHB (red) and class 3 EHB (blue), with the latter highlighting that members of the same 16S rRNA operational taxonomic unit can differ markedly in their genomic traits. Boxes containing R and X indicate class 3 EHB that have been reassociated with cured hosts under laboratory conditions (R) and transferred successfully and stably into novel fungal hosts (X). Labels for bacterial endophytes are similar to those of EHB in fungal endophytes but lack fungal hosts, because these bacteria occurred directly in plant tissues. Phylogenetic reconstruction from reference 38 depicts the results of a Bayesian analysis of 16S rRNA gene sequences. Branch support values indicate parsimony bootstrap values (≥70%; before slash) and Bayesian posterior probabilities (≥95%; after slash). Branches in bold indicate ≥70% neighbor-joining bootstrap values. Endohyphal Burkholderia listed here have since been reclassified as Paraburkholderia (79).
and that they vary reliably in suites of characteristics that can be used to place them into an operational classification. Below we define a preliminary classification for EHB before exploring in greater detail their functional significance. We then focus on one functional group to explore methods of study and research frontiers.

**FUNCTIONAL CLASSIFICATION, TRAITS, AND SIGNIFICANCE OF EHB**

When BLOs were first described from hyphae and spores of various Glomeromycotina, three interpretations were put forward with respect to their potential functional significance: (i) they might be obligate symbionts living in close and sustained relationships with their host fungi, (ii) they might be transient associates, repeatedly lost and reacquired by fungi, and/or (iii) they might be organelles of uncertain function and origin (68). Evidence now supports the first two interpretations for diverse EHB, albeit in different functional groups of fungi and bacteria. Here we assign a functional classification to EHB that references the phylogenetic classifications and functional traits of host fungi, genomic traits of EHB, and corresponding ecological and evolutionary traits of EHB such as transmission mode (Table 1). We identify three functional classes, here defined as class 1, class 2, and class 3 EHB.

**Class 1 EHB**

Class 1 EHB (Table 1) correspond to the EHB typically referred as Mollicutes-related endobacteria. These are consistent with the small, coccoid BLOs observed over the past half-century in arbuscular mycorrhizal fungi (41, 42, 68, 76, 87–90). These members of the phylum Tenericutes are closely related to various Mycoplasma species in the class Mollicutes (41, 42) and are thought to represent ancient associates of Glomeromycotina and related fungi (42, 87). Recently, a genus was erected to encompass these EHB (Candidatus Moeniplasma) (91). They have not yet been isolated in culture (91). The endohyphal Burkholderia listed here is now Paraburkholderia (79).

**Figure 5** Context-dependence in the outcomes of interactions between class 3 EHB and foliar endophytes (rows) and meaningful phenotypic variation among symbiotic partners (columns). Cells reflect the significance and directionality of repeated-measures analyses of variance assessing growth of fungal strains containing EHB and clones that were cured of EHB by antibiotic treatment *in vitro* over 14 days on water agar (low nutrient), malt extract agar (high nutrient), lignin medium (with indulin as the sole carbon source), and cellulose medium (carboxymethylcellulose as the sole carbon source). Thermotolerance was assessed on two media at 36°C. Cellulase activity was measured as zone-of-clearing scaled by colony diameter. Orange cells indicate that the growth or cellulase activity of clones with EHB significantly exceeded that of cured clones. Blue cells indicate that the growth or cellulase activity of cured clones significantly exceeded that of clones with EHB. Gray boxes indicate no significant difference (ns) as a function of EHB status. Fungi in red differ only in the identity of their EHB, in that their fungal genotypes at the barcode locus are identical. The endohyphal Burkholderia listed here is now Paraburkholderia (79).
Mycoplasma (90). Genomes of Mollicutes-related endobacteria sequenced to date are characterized by low GC content (Table 1), low gene content, and proteins with domains involved in interactions with eukaryotic hosts, as well as genes acquired by HGT from arbuscular mycorrhizal fungi (41, 42). Genes acquired from their hosts include protein domains relevant to fungal programmed cell death, self/nonself recognition, and posttranslational modification (summarized in reference 90). Their genomes also show evidence of intimate interactions with plectroviruses (Inoviridae, single-stranded DNA viruses affiliated with diverse Tenericutes [41]).

At present there is some debate regarding the functional roles of class 1 EHB. Their metabolic reliance on their fungal hosts is underscored by their limited biosynthetic capacity (summarized in reference 90). Genome analyses designed to infer function are hindered by the genetic distinctiveness of class 1 EHB, because functional annotation is lacking for >50% of genes (90). The conclusions of two contemporaneous papers differ with respect to the potential for class 1 EHB to be antagonists or mutualists with respect to their host fungi (41, 42, 88).

Class 2 EHB
In terms of host breadth, genome size, genomic traits, obligacy, and metabolic reliance on their hosts, the diverse assemblage here identified as class 2 EHB is intermediate between classes 1 and 3 (Table 1). These bacteria associate with diverse Mucoromycota and Ascomycota (Table 1). Members of class 2 demonstrate a capacity for transmission via spores as well as growth in vegetative hyphae and reproductive structures such as ascosporas (e.g., in the ectomycorrhizal fungus Tuber borchii) (15, 93, 94). They are best known in soilborne pathogens, seed-infecting fungi, ectomycorrhizal fungi, and the arbuscular mycorrhizae in which they were first characterized.
Class 2 EHB have moderately sized genomes (ca. 1.4 to 3.8 Mb), moderate GC content (ca. 46 to 60%), a predominance (although with some exceptions) of vertical transmission, limited culturability or a high degree of fastidiousness, and in many cases, occurrence in fungal cells within membranes of host origin (Table 1) (15, 89). Some EHB in this class have yet to be isolated in culture (see 19, 93–95), but some can be cultivated outside of their hosts under laboratory conditions (93). Homogenizing mycelia and using genomics to predict substrate use or optimize growth conditions may greatly increase the success with which EHB in this functional group can be isolated in culture (96).

As presently defined (Table 1), class 2 EHB include members of the Burkholderiaceae (Betaproteobacteria) such as “Candidatus Glomeribacter gigasporum” in various Glomeromycotina (95, 97, 98), Paraburkholderia rhizoxinica and Paraburkholderia endofungorum in R. microsporus (Mucoromycotina [99–101]), and Mycoavidus cysteinexigens in Mortierella elongata (Mortierellomycotina [44, 96]). Also included here are intriguing examples of vertically transmitted and/or apparently unculturable associates of Dikarya, including the Cytophaga-Flexibacter-Bacteroides clade in ectomycorrhizal T. borchii (Ascomycota) (94, 102) and Chitinophaga sp. (Bacteroidetes) in a seed-associated Fusarium keratoplasticum (Ascomycota) (19, 49).

Such class 2 EHB illustrate the range of genomic and ecological traits that characterize this diverse functional group. Here we explore the four best known of these: “Candidatus Glomeribacter gigasporum,” P. rhizoxinica, M. cysteinexigens, and Chitinophaga sp.

“Candidatus Glomeribacter gigasporum” (Burkholderiaceae) is an EHB affiliated with primarily Glomeromycotina such as G. margarita, Scutellospora persica, and Scutellospora castanea (97). It is vertically transmitted through spores and has not been grown in culture outside of its hosts (103), although it can be separated from host mycelium and maintained for several weeks under laboratory conditions (98). Consistent with vertical transmission and a high degree of metabolic dependence on its host, it has a relatively small genome (1.4 Mb with one plasmid [98]; 1.72 Mb with three plasmids [93]) and limited capacity for carbon catabolism, gluconeogenesis, phosphate transport, and the synthesis of essential amino acids. In addition to synthesizing diverse metabolites, including antibiotics, vitamin B<sub>12</sub>, and molecules relevant to resistance against toxins (93), it influences the protein expression and lipid profiles of its host fungus, as well as spore morphology and hyphal growth in presymbiotic phases (104, 105). Like a related member of class 3 EHB (Paraburkholderia sp. 9120; see Fig. 8 and discussion below), it expresses type II and type III secretion systems (43, 93).
Figure 8  Genomic traits of class 3 EHB associated with foliar endophytes in the Ascomycota (blue font) compared with nonendohyphal relatives (black) and a model class 2 EHB (red), showing the bacterial habitat, genome size, gene count, and results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway searches to identify bacterial pathways involved in signaling between bacteria and eukaryotes (43). Boxes along the x axis indicate KEGG pathway identifiers (top) for constituent genes for each bacterial secretion system. Colored boxes indicate that at least one gene within the genome is present and classified according to that specific KEGG identifier. Numbers inside the colored boxes denote that more than one gene within that genome is classified according to that KEGG identifier. Boxes for EHB bacteria described first in reference 43 are shown in blue. Green boxes highlight a species that interacts with fungi but does not appear to occur endohyphally. Endohyphal *Burkholderia* listed here are now reclassified as *Paraburkholderia* (79).
Table 1  Functional classification of endohyphal bacteria into three operational classes based on host information, bacterial phylogeny, genomic traits, and associated traits relevant to bacteria/fungi interactions and ecology (references listed in text)

<table>
<thead>
<tr>
<th>Class</th>
<th>Equivalency or example&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fungal clade</th>
<th>Bacterial clade</th>
<th>Genome size (Mb)</th>
<th>GC content (%)</th>
<th>Transmission</th>
<th>Obligacy of bacterial association</th>
<th>Metabolic reliance on host</th>
<th>Localization</th>
<th>Cell wall</th>
<th>Proposed interaction with host</th>
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<tbody>
<tr>
<td>Class 1</td>
<td>Mollicutes-related endobacteria (MREs)*</td>
<td>Mucoromycota (Glomeromycotina, Mucoromycotina)</td>
<td>Tenericutes</td>
<td>0.7–1.2</td>
<td>32.0–34.3</td>
<td>Vertical</td>
<td>Obligate</td>
<td>High</td>
<td>Cytoplasm</td>
<td>No</td>
<td>Debated and presently unknown</td>
</tr>
<tr>
<td>Class 2</td>
<td>Burkholderia (Paraburkholderia rhizoxinica‡ and Paraburkholderia endofungorum‡, “Candidatus Glomeribacter gigasporum”‡, Chitinothaga sp., EHB01‡, Cytophaga-Flexibacter-Bacteroides‡)</td>
<td>Mucoromycota, soilborne Ascomycota</td>
<td>Proteobacteria, Bacteroidetes</td>
<td>1.4–3.8</td>
<td>46.1–60.7</td>
<td>Vertical but potentially horizontal in some species</td>
<td>Obligate to facultative</td>
<td>Moderate to high</td>
<td>Cytoplasm; some surrounded by membrane of fungal origin</td>
<td>Yes</td>
<td>Generally beneficial but can be context-dependent</td>
</tr>
<tr>
<td>Class 3</td>
<td>Luteibacter sp. 9143‡, Erwinia sp. 9145‡, Pantoea sp. 9140‡, Pantoea sp. 9133‡, Massilia sp. 9096‡, Rhizobium sp. 9140‡, Curtobacterium sp. 9128‡, Paenibacillus sp.¶, Rhizobium radiobacter‡</td>
<td>Diverse Ascomycota, Basidiomycota</td>
<td>Proteobacteria, Firmicutes, others</td>
<td>3.9–8.5</td>
<td>55.2–70.6</td>
<td>Horizontal</td>
<td>Facultative</td>
<td>Moderate</td>
<td>Cytoplasm; some surrounded by membrane of fungal origin</td>
<td>Yes</td>
<td>Mixed; highly context-dependent</td>
</tr>
</tbody>
</table>

<sup>a</sup>*, equivalency; ‡, example.
Ghignone et al. provide a model for tripartite interactions among “Candidatus Glomeribacter gigasporum,” its fungal host, and the host plant with which these symbionts interact, highlighting energy fluxes in living cells, metabolic exchange, and the occurrence of the EHB in protein-rich vacuoles (93). This species is a model for studies of plant-arbuscular mycorrhizal-EHB associations.

Paraburkholderia rhizoxinica (formerly Burkholderia rhizoxinica) is affiliated with R. microsporus (Mucoromycotina), in which it is responsible for producing the toxins that have been identified as causal in rice seedling blight disease (14, 52). These include rhizoxin, a potent antimitotic agent, and rhizoxin, a hepatotoxic cyclopeptide, both of which were previously thought to be mycotoxins produced by R. microsporus (52, 99, 101). Interestingly, the fungal host requires P. rhizoxinica for sporangium and spore development, tightly linking EHB with fungal reproduction and fitness (100). The same authors developed techniques for labeling and artificially introducing the bacterium into fungal structures, thus establishing a highly informative experimental system for studying EHB-fungi interactions (100). By studying resistance of fungi to the antimitotic properties of rhizoxin, Schmitt et al. suggested that the stable affiliation of P. rhizoxinica with R. microsporus represents a transition from parasitism to mutualism (106). Consistent with its close association with its fungal host, P. rhizoxinica has a 3.75-Mb genome with primary metabolism oriented to the acquisition and use of fungal metabolic products (107). Like many other EHB, and many non-EHB in the Burkholderiaceae, it is characterized by the presence of multiple plasmids in addition to its own chromosome. The biosynthetic gene cluster relevant to rhizoxin is located on the bacterial chromosome itself (107). Although it is transmitted via spores, evidence from a global population study suggests that past horizontal transmission events have shaped the genomic architecture of this species (47). P. rhizoxinica can be grown with limited success in culture under laboratory conditions (47, 52, 107). This EHB can actively invade fungal hyphae in part through use of a type II secretion system, which releases chitinolytic enzymes (107, 108). Strikingly, incursion into fungal cells occurs via softening of the fungal cell wall and leaves little detectable trace (108). In pure culture the EHB is motile, occurring in pairs or at times in clusters as short or coccolid rods ca. 1.2 to 2 μm long and 0.6 to 1.2 μm in diameter (107). This EHB was the first to be characterized as markedly altering the phenotype of fungi with respect to plant pathogenicity and toxin production (14), and it is a model system for genomics and the study of plant pathogen-EHB interactions. A close relative, Paraburkholderia endofungorum, was described in 2007 and shares many traits with P. rhizoxinica (109).

Mycoavidus cysteinexigens (Burkholderiaceae) is an EHB associated with the soil-inhabiting fungus Mortierella elongata (Mortierellomycotina) (44, 63, 110). This bacterium has been studied primarily via metagenomics (44) and genome sequencing of the bacterial fraction released from homogenized or macerated mycelium (110). Its 2.6-Mb genome reflects reduced function and considerable reliance on the fungal host (111). Detection of a potential cysteine dependency from genomic analyses led to its successful isolation (110), and treatment with antibiotics has revealed that it plays a major role in modulating host metabolism (44). The association between Mortierella and Mycoavidus, like that between other EHB and Glomeromycotina, is thought to be ancient, spanning in this case an estimated 350 million years (44).

Unlike the above examples, which encompass EHB in the Burkholderiaceae that occur in fungi in the Mucoromycota, Chitinophaga sp. EHB01 (Bacteroidetes) is affiliated with a member of the Ascomycota. It was isolated originally from F. keratoplasticum PS0362A, which in turn was isolated from a seed of a tropical tree (Cecropia insignis) after exposure to soil for several weeks in a lowland forest understory (19, 49). The genome of this EHB has not yet been sequenced, but the relevance of the Chitinophaga-Fusarium association has been evaluated using phenotypic microarrays. Since their development, phenotypic microarrays have been extended from evaluations with bacteria only to studies of yeasts and filamentous fungi. Shaffer et al. used phenotypic microarrays to compare growth of F. keratoplasticum with and without Chitinophaga on diverse carbon substrates (19). The fungus grew readily on standard growth media and on diverse substrates in the absence of the EHB, and on standard media, colony diameter and spore production were similar regardless of the presence of the EHB. However, global substrate use (i.e., growth across all substrates considered simultaneously) differed markedly between clones as a function of Chitinophaga infection. In particular, the axenic fungus grew to a higher density over the course of the experiment on 5 (of 95) carbon substrates (1 mono-saccharide, D-arabinose; 2 stereoisomeric forms of one carboxylic acid, D- and L-malic acid; and 1 amide, succinic acid). However, with its EHB, the fungus grew to a higher density on 59 of 95 substrates, including >75% of sugar-based substrates and most amino- and carboxylic acids and their derivatives (19).
Thus, *Chitinophaga* EHB01 is associated with increased breadth of substrate use and enhanced growth by its host on many substrates. Moreover, its improvement of fungal growth on substrates relevant to plant and seed-fungus interactions is intriguing from an ecological standpoint. Much as genomic insights can be used to infer phenotypic traits, here phenotypic observations provide the basis for predictive genomics, currently a target for future work. Although vertical transmission of *Chitinophaga* has not yet been observed, we anticipate that challenges associated with isolating it in culture will correspond to a relatively small genome that is tightly integrated with host functional traits. This argues for its placement in class 2, much like the *Cytophaga-Flexibacter-Bacteroides* symbiont of the ectomycorrhizal ascomycete *T. borchii* (102). However, genomic data are needed for these strains, which stand out from other class 2 EHB by occurring in septate fungi within the Dikarya, rather than in the coenocytic Mucoromycota described above. These Ascomycota-associated *Bacteroidetes* may prove to be a unique functional group or may be moved to class 3 should cultivation be achieved and transmission by spores not be detected.

**Class 3 EHB**

Class 3 EHB are a diverse, nonmonophyletic assemblage of bacteria characterized primarily by horizontal transmission, partially free-living or otherwise non-endophyhal lifestyles, high phylogenetic diversity, and occurrence in the living hyphae of Dikarya (11, 17, 19, 38, 43, 48, 49, 85, 112). As a group these EHB represent the second scenario outlined by MacDonald et al.—that EHB may be transient associates of their hosts, with the capacity to be gained and lost repeatedly (68; see also 38, 48). Such gains and losses are easily detectable in the laboratory and speak to a facultative lifestyle in nature (17, 38, 48, 49).

Class 3 EHB as defined here occur in hyphae of Ascomycota and Basidiomycota affiliated with soil and above-ground plant parts. Hosts include ectomycorrhizal *Laccaria* (*Basidiomycota* [84]), growth-promoting root endophytes such as *P. indica* (*Basidiomycota* [85]), and diverse *Pezizomycotina* that occur in leaves and seeds of plants from tropical to arctic settings (38, 49). Together, class 3 EHB represent at least the phyla *Proteobacteria* (including *Alpha-, Gamma-,* and *Betaproteobacteria*; Fig. 4) and *Firmicutes* (e.g., *Paenibacillus* in *Laccaria* [112]; see also reference 38 for a report of *Paenibacillus* in *Phaeomoniella* and *Biscogniauxia* strains that occur as foliar endophytes).

The bulk of the diversity in this functional group appears to reside in the Proteobacteria, represented by genera such as *Paraburkholderia* (with large genome sizes compared to congeneric bacteria occurring as class 2 EHB [Fig. 4]); *Rhizobium* and *Acinetobacter*, associated with *Sebacinales*; and *Luteibacter*, *Pantoel Erwinia*, *Sphingomonas*, and others (Fig. 4; Table 1). Generally, these bacteria are closely related to free-living or other non-EHB lineages (38, 43). They can be visualized using LIVE/DEAD staining and fluorescence in situ hybridization (Fig. 2). In some cases the number of bacterial cells per fungal cell can be quite low (e.g., 2 to 20 per fungal cell [113]). The few cases in which bacterial DNA has been quantified with respect to fungal genomic DNA reveal concomitantly low amounts of bacterial genomic material (e.g., 0.035 ng of bacterial DNA:100 ng of fungal DNA [114]).

Class 3 EHB typically can be cultured outside of their hosts, reintroduced to their axenic hosts, and moved into novel fungal strains (Fig. 3) (17), presenting a tractable set of experimental systems for tracking the relevance of EHB in modulating fungal phenotypes (11). At least some species are encapsulated in the cytoplasm of Dikarya by membranes apparently of host origin (M. Hoffman, personal communication). Individual fungi can harbor more than one class 3 EHB in different parts of the same mycelium (49), with EHB transformation allowing visualization of single- and multi-EHB infections (see Fig. 3).

Class 3 EHB appear not to have undergone significant reductions in genome size or content relative to their close relatives with other lifestyles. The genomes of these EHB generally are larger than those of class 1 and class 2 EHB (Table 1; Fig. 4), with higher GC content and a prevalence of genomic traits consistent with life phases outside of fungal hyphae (43). Their genome sizes range from ca. 3.9 to 8.5 Mb, with GC content exceeding 70% in some strains (Table 1; Fig. 8). Most have circular chromosomes and varying numbers of plasmids, but *R. radiobacter* has both a circular chromosome and a linear chromosome (together accounting for 4.9 Mb) as well as one plasmid (0.21 Mb) (114). GC content (ca. 59.3 to 59.4% in the bacterial chromosome) is similar to that of many other class 3 EHB (Table 1). Like other class 3 EHB, *R. radiobacter* is closely related to bacteria that do not occur as EHB (in this case, to plant-pathogenic bacteria [114]).

Preliminary evidence indicates profound effects of individual class 3 EHB on their fungal hosts with respect to altering phytohormone production, thermotolerance, substrate use, and the production of plant cell wall-degrading enzymes (Fig. 5) (see also references
FRONTIERS FOR STUDYING EHB

The study of EHB was initiated when observations of fungal structures by microscopy revealed the presence of unexpected structures resembling bacteria in the cytosol (53, 66, 68, 76). Since that time inquiries into the existence, identity, and function of EHB have followed a trajectory that includes imaging; curing and, where possible, reinfection; phylogenetic characterization; and most recently, genomics and metagenomics that have in some cases facilitated culturing and in other cases illuminated the evolutionary history of uncultivable species (41, 42, 88). In the near future metatranscriptomes will be exciting for understanding gene expression, particularly in class 2 and class 3 EHB whose relationships with fungi can be manipulated. Variation in the outcomes of associations between class 3 EHB and different fungi (Fig. 5) sets up an especially compelling area of inquiry in which metatranscriptomic perspectives will be enlightening. Those fungus-EHB pairs that can be cured and reassOCIated, or that can be introduced easily into other fungi, might be promising targets for such work (see Fig. 3, Fig. 4, Fig. 5).

In the interim, current and traditional methods of evaluating fungi for EHB continue to be fruitful. The prevalence of class 3 EHB in the species-rich Dikarya argues for screening existing fungal collections and new isolates from diverse phylogenetic and functional groups to understand the scope and distribution of EHB symbioses. At the moment, most known EHB are in plant-associated fungi; EHB (class 2) have been detected in clinical isolates of R. microsporus, but the bacteria are not required for zygomycoses (47). Given the importance of such EHB as producers of toxins, however, an important area for future research relevant to human health certainly lies in food safety (e.g., in the context of Paraburkholderia affiliated with Rhizopus strains used for tempeh production; see references 47, 117).

In the earliest studies that detected EHB, transmission electron microscopy was key for diagnosing the presence of BLOs in hyphae of arbuscular mycorrhizal fungi (53). A decrease in the training of young mycologists in transmission electron microscopy and related methods (including ultrastructural analysis and other imaging approaches such as scanning electron microscopy) may limit visualizations that so richly complement -omics studies of EHB. In the case of class 3 EHB, resynthesis conditions have been published for the association between Pestalotiopsis and Luteibacter, outlining an approach for manipulating EHB and their partners that could be translated to microscopy and linked directly to genomics/metatranscriptomics analyses (48). In seeking to link structures with genes and gene expression, mycologists may benefit in some cases from collaborations: a survey of the literature reveals...
that few papers on EHB have been published in mycological journals, suggesting untapped potential to link fungal biology with the work of microbial and genomic scientists outside the traditional scope of mycology.

At the end of the 20th century, intracellular *Para-*burkholderia (class 2) were described in the cytoplasm of Glomeromycotina through the combined approaches of PCR and fluorescence microscopy (e.g., 46). Staining spores of *G. margarita* with LIVE/DEAD (46) confirmed the viability of both the fungal and bacterial symbionts, a much-needed indication of symbiosis that should be established clearly when new EHB-fungi associations are described (see 15). Similarly, Salvioli et al. used SYTO BC dye, a nucleic acid stain component, in conjunction with confocal microscopy to detect “*Candidatus* Glomeribacter gigasporum” in spores of *G. margarita* (13). Such methods persist in more recent studies as effective approaches for validating the presence and placement of living bacteria in diverse fungi (38, 48, 49). However, methods such as LIVE/DEAD can lead to uncertain interpretations: fluorescence by ectophyphal bacteria and fungal nuclei and mitochondria cannot always be distinguished easily from EHB. In these cases, combining molecular analysis and more specific methods such as fluorescence *in situ* hybridization can be helpful. For example, Hoffman and Arnold used phylogenetic approaches based on 16S rRNA in conjunction with a TAMRA fluorophore to visualize and unambiguously confirm the identity of class 3 EHB (Fig. 2) (38).

Here, too, transformation of EHB can be of immense help in visualizing bacteria within fungal cells. For example, Partida-Martinez et al. used green fluorescent protein labeling to track vertical transmission of class 2 EHB (100). Similarly, Arendt et al. imaged tdTomato-tagged *Luteibacter* sp. 9143 in hyphae of its host (48). In the latter study, the *Luteibacter* strain was manipulated with transposon mutagenesis (Fig. 3) (48). In such cases, EHB that can be grown axenically, be manipulated, and be reintroduced to hosts can be especially tractable.

In general, class 3 EHB can be isolated from fungal hyphae after prolonged immersion in water, after heat treatment, or after macerating or otherwise damaging fungal mycelia (an approach that also has been useful in segregating some class 2 EHB for molecular analysis [96]). The emergence of EHB in culture, particularly from vouchers of fungi such as endophytes stored in sterile water, sometimes can be mistaken for contamination if their presence is unexpected (38). Occasionally, EHB may overcome fungi vouched in water, making it impossible to regrow stored fungal strains. In such cases, fungaria may do well to voucher dual cultures: those grown on antibiotic-containing media and those grown on standard media (38). If fungi are originally isolated from the environment using antibiotic-containing media, EHB may be removed before downstream studies occur, and in such cases vital aspects of fungal traits—shaped by their EHB—might be overlooked. Because some fungi may harbor more than one EHB strain concurrently (49), future work should incorporate next-generation sequencing methods to evaluate the diversity and composition of EHB communities in individual mycelia.

More generally, a wealth of new or newly applied methods provides a compelling opportunity to address questions that are fundamental to understanding the traits and importance of EHB. Here we explore these with respect to molecular approaches, imaging, and questions of interdomain communication. In the following discussion we focus primarily on EHB that can be isolated and grown in culture, which are tractable for studying the initial phases of bacteria-fungi interactions, their chemical currency, their genetic and phenotypic responses to one another, the timeline of their associations, and—as demonstrated elegantly by Moebius et al. (108)—the dynamics of bacterial colonization into fungal hyphae. In this context, approaches used to study bacteria-fungi interactions in nonendo-fungal systems can shed light on principles of interdomain associations and their outcomes in the context of EHB symbioses. We explore several such avenues, below especially with regard to frontiers in the study of class 3 EHB, a ubiquitous group of symbionts with horizontal transmission that appears widespread in the most species-rich fungal clades.

**Frontiers in Transcriptomics and Genomics**

As the cost of high-throughput sequencing drops and more genomic data become available, opportunities will increase to understand the genetic underpinnings that shape bacteria-fungi interactions at stages ranging from first encounters to symbiont-driven modulation of fungal traits. In particular, transcriptomics tools have become increasingly important for understanding how genes are regulated during biotic interactions. For example, by comparing transcriptomes of the fungus *Aspergillus niger* and the bacterium *Bacillus subtilis* during coculture and axenically, Benoit et al. observed obvious metabolic changes upon attachment of the bacteria to fungal hyphae (12). Both *B. subtilis* and *A. niger* decreased their production of antifungal and antibacterial molecules, respectively. The majority of the genes downregulated during these *in vitro* inter-
actions were related to detoxification or secondary metabolite production and carbon metabolism, suggesting that an active physiological adaptation to the partner organism occurred in culture (12). Such dynamism may underscore facultative associations between class 3 EHB and their fungal hosts.

Similarly, Mela et al. used a dual transcriptomics approach to explore bacterial genes involved in mycophagy by *Collimonas fungivorans* and the fungal response to this attack (118). Their findings indicated a complex interaction involving antibiosis and nutrient competition in both organisms. On the bacterial side, chitinases and secondary metabolites played an important role. The fungal response was dominated by differential expression of genes related to cell wall and lipid degradation, nitrogen deficiency, and cell defense. Future studies could evaluate whether fungi exhibit similar responses when exposed to their native EHB (as opposed to EHB from other fungi) or when exposed to other bacteria that are closely related to EHB yet have other lifestyles (see 17).

Although there are commonalities in the fungal response to antagonism in both of these studies, common ground also exists between positive and negative interactions. A dual-transcriptomics microarray study of the interaction between *L. bicolor* and three bacteria (a helper strain, a commensal strain, and an antagonistic strain) revealed negative correlations in transcripts involved in primary metabolism between antagonistic and nonantagonistic interactions (119, 120). However, the fungus had a unique response to each bacterial strain. Although each bacterial strain responded differently to the fungus, all three bacteria demonstrated differences in the expression of genes involved with cell wall modification (119, 120). The relatively small size of microbial genomes and differences in mRNA processing between prokaryotes and eukaryotes make bacteria-fungi interactions ideal for transcriptome studies, with the potential to explain how EHB modulate the phenotypes of their fungal hosts in real time.

More broadly, the accumulation of genome-scale data from diverse EHB over the past 2 decades promises to accelerate as these associates of fungi, and their relevance, become better known. Comparative genomics for densely sampled clades that contain EHB has the potential to identify hypotheses regarding mechanisms of interactions between fungi and their endosymbionts. Variation in the presence of various protein secretion systems (e.g., Fig. 8) suggests that as a whole, EHB may communicate and interact with their fungal hosts in highly diverse ways. Chitinase has proved especially important for facilitating bacterial entry into living fungal hyphae (108), but it is striking that genomes of diverse EHB do not necessarily contain genes for chitinase production (Fig. 8) (43). Studies regarding alternative methods of entry into fungal hyphae merit further attention.

One related area of inquiry that also merits exploration is that of HGT between EHB and their fungal hosts. Illustrated clearly in the case of class 1 EHB (41), HGT contributes greatly to bacterial genome evolution in general (121) and has been detected, albeit at a lower frequency, in diverse fungi (122). Several genomic studies have suggested HGT between bacteria and fungi (123, 124), a process that could be accelerated or increased in frequency when fungi host EHB. We anticipate that HGT may be especially common in EHB with type IV secretion systems similar to that of *Agrobacterium*, which have been used to successfully transform fungi *in vitro* (125). Genes encoding for type IV secretion systems have been observed among diverse EHB in class 2 (e.g., plasmid-encoded in *P. rhizoxinica* [107]) and class 3 (Fig. 8) (43).

### Frontiers in Metabolomics

Many new tools for studying signaling molecules relevant to bacteria-fungi interactions are increasingly available and can be applied to the study of EHB. One area of interest lies in the production and relevance of volatile organic compounds (VOCs) (see “Frontiers in Interdomain Communication,” below). For example, recent advances in mass spectrometry (MS) acquisition and analysis have helped to elucidate VOCs used as novel communication signals of soilborne bacteria and fungi. Solid-phase microextraction devices are small filaments that can be inserted into the airspace of interest to adsorb VOCs and then be analyzed directly in gas chromatography MS. These devices have been used with cocultures as well as directly on substrates to identify compounds mediating intermicrobial interactions (126–128). Profiling of VOCs also is being advanced through tools such as the electronic nose, or e-nose, which does not provide specific identities of compounds in sampled air but provides entire profiles that allow researchers to make global comparisons between samples or to detect specific microbial profiles in the environment (129–132). To our knowledge, this method has not been used to examine specific bacteria-fungi interactions per se, though it has been used to profile the progression of truffle aromas over time (129), which are thought to be attributable to the fungus as well as its bacterial associates (133).

In turn, diffusible interactions are a related area of interest for exploring presymbiotic phases of EHB-
fungi interactions. Plating colonies in opposition, then extracting and assaying metabolites to examine outcomes (i.e., antibiosis, growth stimulation, chemotaxis) has been the “gold standard” in this area for some time. However, many recently developed techniques are extraction-free, which helps reduce inherent bias of extraction methods and gives a more complete picture of the molecular interplay of microbial interactions. Imaging MS is an especially promising platform, because it allows researchers not only to determine what compounds are in a sample, but also to map the location of those compounds to specific locations in co-culture (134). One such method, matrix-assisted laser desorption imaging MS (MALDI-MS), is an extraction-free method that can assess the mass spectral patterns of interacting microbes on solid media. Matrix-assisted laser desorption imaging MS was used to identify ralsolamycin, the interdomain communication compound that induces formation of fungal chlamydomospores (135), and to map metabolic interactions and conversion of bacterial phenazines by Aspergillus fumi-gatus (136). Finally, nanospray desorption electrospray ionization MS provides the ability to analyze metabolites directly from living environmental samples at the microscale (137, 138). When these techniques are combined with metabolic networking platforms, metabolic exchanges can be identified (139) that could inform presymbiotic and early interactions between EHB and their fungal hosts.

Frontiers in Imaging
Electron microscopy has a long and fruitful history with regard to illuminating how diverse bacteria interact with and occur in fungal hyphae (e.g., 68, 108). For example, Levy et al. used electron microscopy to explore colonization of Giga-spora decipiens spores and hyphae by a Paraburkholderia sp. (class 2 EHB) (140). Their work revealed previously undescribed fibrillar structures that were used to adhere to the hyphal surface. In parallel, improved spatial resolution has made confocal microscopy similarly indispensable for exploring close associations of bacteria and fungi, especially in conjunction with fluorescence in situ hybridization and related approaches (see 141). In both class 2 and class 3 EHB, interactions between bacteria and fungi can be temporally dynamic, such that monitoring these interactions as they progress is desirable (see 108). While traditional time-course studies have set the groundwork for understanding general processes in bacteria-fungi interactions (12), new tools can allow continuous, nondestructive monitoring of interactions through time. Stanley et al. recently used novel micro-

fluidic devices to monitor interactions continuously between Coprinus cinerea and B. subtilis, showing dynamics in attachment, growth rates, and cell death resulting from these interactions (142). Such approaches provide an exciting new avenue for exploring microbial interactions over time at a microscopic level, including the dynamics of colonization by EHB, potential cell-sorting or selection of symbionts by fungi, and the abundance of colonists under particular conditions.

Frontiers in Interdomain Communication
Directed signaling between bacteria and fungi in environments such as soils often relies on VOCs (143). These compounds are generally lipophilic and have a low molecular weight, high vapor pressure, and low boiling point, which facilitates their movement through substrates via vapor diffusion and advection (i.e., matter transfer via fluid). Recent work demonstrates that VOCs are produced frequently by bacterial symbionts of plant-associated and soilborne fungi, with effects on fungal morphology, growth, and persistence in the environment (144). Such effects can be positive or negative and have strong impacts, especially at the spore germination and mycelial growth phases of fungal life cycles (145, 146).

In turn, some bacteria and fungi participate in two-way signaling. When the bacterium Ralstonia solana-cearum was cocultured with a plant-pathogenic strain of Aspergillus flavus, conidiation by A. flavus decreased and aflatoxin production increased (127). At the same time, R. solanacearum showed decreased growth, decreased melanin production, and extracellular polysaccharide production (127). Sampling of the airspace of these cocultures and axenic cultures revealed VOC profiles that could influence autoinduction systems. We anticipate that further exploration of microbial signaling will show that VOCs contribute to subtler shifts in physiology than those described to date, which is potentially important in the early phases of communication between EHB and their fungal hosts.

As the above studies suggest, fungi are likely active participants in establishing interactions with EHB and, like many hosts that harbor microbial symbionts, play some role in filtering colonization by microorganisms. One particularly exciting area for future work with respect to such close interdomain interactions might focus on lectins. These proteins that react with sugars represent one of the few examples in which fungal activity is known to be influential in the early stages of interactions with bacteria. Lectins are widespread in fungi. They can mediate interactions between bacterial
and fungal pathogens and their hosts, including both plants and animals (147). Although reported primarily from Basidiomycota (148), lectins with relevance to bacteria-fungi interactions are known in some Ascomycota. For example, Díaz et al. showed that a glycosylated arginine acting as a lectin from the lichen-forming fungus Peltigera canina was involved in recruitment and attachment of cyanobacterial cells to the mycobiont (149). Given that both fungal and bacterial cell walls are extensively decorated with diverse carbohydrate moieties, lectins seem to be a reasonable area of focus for exploring the early signaling, chemical interactions, and attachment relevant to the establishment of EHB.

Similarly, oxalic acid is produced by members of diverse fungal phyla (150). Oxalic acid is continuously excreted from hyphal tips, sometimes in the form of oxalate crystals. Mycophagous Collimonas uses oxalic acid to locate hyphal tips (150). Bacterial motility experiments demonstrated that Collimonas can recognize and engage in directed growth based on concentrations of free oxalic acid (150). It is plausible that this and similar signaling molecules may be important in the initiation of EHB symbioses.

More generally, such observations speak to an important interplay of chemotaxis, extrahyphal localization, and early symbiotic interactions between bacteria and fungi (119). L. bicolor uses chemotaxis to attract the mycorrhizal helper bacteria Pseudomonas fluorescens (119), selecting for particular strains by exuding trehalose, a polyol compound that is a carbohydrate source in and around the mycorrhizal association. When trehalose is used as the sole carbon source in thin agar on glass microscope slides, fluorescent P. fluorescens bacteria accumulate onto and around the hyphae. This kind of attraction of the bacteria specifically to hyphae by the fungus indicates the potential for close interaction and communication, possibly signifying nutritional exchanges (119). Such signaling would be of interest in the case of class 3 EHB and in those cases in which de novo colonization occurs in fungal hyphae by class 2 EHB.

Once in proximity, bacteria and fungi typically communicate over shorter distances (micrometers to millimeters) via larger (>300 daltons), nonvolatile, and often specialized signals. These signals can be proteinaceous or nonproteinaceous and can diffuse through aqueous environments. We anticipate that proteinaceous signaling molecules are particularly important in pre- and early symbiotic phases of EHB-fungi associations. Fungi and many bacteria acquire nutrients via extracellular digestion, such that they secrete digestive enzymes into their local environment. These enzymes include cellulases, chitinases, glucanases, proteases, and lysozymes (151, 152). Although little has been documented about the roles of enzymes in direct bacteria-fungi interactions, chitinases have been indicated as important factors for bacterial mycophagy (152) and EHB entry into fungal cells (108).

Frontiers with Respect to Protein Secretion Systems

Bacteria rely on secretion systems to release large biomolecules into the local environment and neighboring cells. These systems can range from simple transporters to large protein complexes with specialized functions. In Gram-negative bacteria these multicomponent secretion systems have been catalogued into at least six types (type I to type VI), each differing in components and molecular mechanisms. These systems often are associated with virulence but have also been implicated in other symbiotic associations. They certainly are relevant in many EHB-fungal associations, but studies in this area are in an early phase.

Type I and type II systems secrete lipases, proteases, and beta-glucanases, which have been implicated in antifungal properties of different bacterial species (153, 154). Type II systems that secrete chitinases are essential for bacterial mycophagy in the Paraburkholderia-Rhizopus endosymbiosis (class 2) (108).

Type III and type IV systems directly inject macromolecules such as proteins and DNA into host cells and have been implicated mostly in interactions with plants and animals, although there is a growing body of literature that suggests that these systems can also impact interactions with fungi. Specifically, type III secretion systems are essential for the maintenance of endosymbiotic bacteria in Paraburkholderia-Rhizopus interactions (107). Similarly, type III systems influence how the mycorrhizal helper bacterium, P. fluorescens BBc6R8, shapes colonization of pine roots by L. bicolor (155). In turn, Agrobacterium tumefaciens (syn. R. radiobacter) utilizes type IV systems to inject transformative plasmids into host plants (156). This transformation system has been adopted for biotechnological purposes to transform a number of fungi in vitro (125). Thus, type IV systems may have a yet undescribed function in mediating the transfer of genetic material from bacteria to fungi. While it is tempting to assume that some evidence of bacteria-fungi HGT may have been mediated by bacterial type IV secretion systems, particularly via EHB symbioses, the mechanisms of such gene transfer events have yet to be explored.
Type V and VI systems have received limited attention to date in EHB, although they may also play a role due to their implications in pathogenesis and interactions with eukaryotes. Type V systems secrete virulence factors and adhesins through the outer membrane, which can contribute to attachment and biofilm formation (157). These systems may be important for maintaining extracellular contact with fungi during early colonization and potentially matter for fungi-mediated translocation. Newly classified type VI secretion systems are important for virulence and are conserved across pathogenic (i.e., *Vibrio*, *Pseudomonas*), beneficial (i.e., *Rhizobium*), and environmental *Proteobacteria* (158). Because of the ubiquity and potential diversity in impacts of type VI systems, their potential roles in EHB associations are compelling for further exploration. Notably, genes for type VI secretion systems are widespread in diverse class 3 EHB (Fig. 8) (43).

Importantly, secretion systems are not the only elements relevant for EHB to enter fungal hyphae. The occurrence of *Firmicutes* and *Bacteroidetes* as EHB opens the door to exploring other means by which class 3 EHB and others with horizontal transfer via an extrasymbiotic phase can gain entry into the living cells of their fungal hosts. In the broad sense, these widespread and potent symbioses appear to be an extremely common feature in the biology of plant-associated fungi. We anticipate that future work will detect additional bacterial phyla and fungal clades involved in intimate interdomain relationships that impact the ecological modes of fungi and the hosts with which they, in turn, interact. Adopting the perspective of Wolf and Wolf (65) argues for assessing fungal holobionts—with their symbiotic bacteria taken into consideration—when exploring the phenotypes, gene expression, communication, and ecological activity of fungi. Together, these are exciting frontiers that have been enabled by a foundation of pioneering studies and the confluence of front-line methods in imaging, biochemistry, phenotyping, computation, and molecular biology.


References


